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# Phosphate-activated glutaminase expression during tumor development

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## Abstract

Changes in phosphate-activated glutaminase activities determined in intact cells and isolated mitochondria have been followed during mouse Ehrlich ascites carcinoma development. Glutaminase activities parallel the levels of poly(A)<sup>+</sup> RNAs encoding for the mitochondrial phosphate activated glutaminase. During the exponential growth phase, maximum activity was observed and the relative abundance of glutaminase mRNA significantly increased with regard to the stationary growth phase. The presented results show that tumor phosphate-activated glutaminase is subject to long-term regulation by differential gene expression.

**Key words:** Tumor glutaminase; Glutaminase expression; Tumor development

## 1. Introduction

Glutamine is avidly consumed by rapidly dividing cells [1]; in fact, glutamine is a good respiratory substrate and a source for nitrogen compounds (for reviews see [2,3]). The first step in glutaminolysis is that catalysed by phosphate-activated glutaminase (PAG) (EC 3.5.1.2), a mitochondrial enzyme [4]. An enzymic imbalance in the glutamine-related enzymes occurs in tumor cells. In human hepatocellular carcinoma, glutamine synthetase activity (EC 6.3.1.2) is one-third of that in normal liver, whereas PAG increases approximately 20-fold [5]. Indeed, PAG activity is correlated with malignancy [6] in tumors and with growth rate in normal cells, e.g. thymocytes [7]. In human diploid fibroblast cultures, Sevdellian et al. [8] reported that the capacity to hydrolysing glutamine reaches a maximum after 2 days of growth and this activity is independent of the glutamine concentration in the culture medium.

PAG is present in different mammalian tissues: mostly in brain, liver, kidney and enterocytes [9]. Two isozymes have been described: liver type and kidney type [9]. In spite of the main role of glutamine metabolism in tumors [2,3], studies on PAG in tumors are scarce. Quesada et al. [10] concluded by analysis of kinetic behaviour and immunoblot analysis that Ehrlich ascitic tumor PAG is a kidney type enzyme. Very recently, Curthoys' group

[11] reported a long-term regulation for rat kidney PAG during metabolic acidosis; in the same way, Smith and Watford [12] reported that liver PAG mRNA increase 4-fold in diabetic rats.

In this study, changes in hydrolysing glutamine capacity of intact cells and isolated mitochondria, as well as changes in phosphate-activated glutaminase mRNA levels, were followed during tumor development; a long-term regulation for tumor PAG expression was deduced.

## 2. Materials and methods

### 2.1. Ehrlich cells and animal treatments

A hyper-diploid Lettré strain of Ehrlich carcinoma was maintained as described elsewhere [13]. Animals were sacrificed on different days after tumor transplantation by cervical dislocation. Immediately thereafter, cells were separated by centrifugation at  $900 \times g$  for 5 min.

### 2.2. Phosphate-activated glutaminase assay in intact cells and isolated mitochondria

Harvested cells were washed three times with 0.1 M Tris-HCl, pH 8, and suspended in the same buffer for 3 h in order to deplete endogenous substrates. Mitochondria were isolated as previously described [14]. The reaction medium was: 0.1 M Tris-HCl, pH 8, 0.2 M potassium phosphate, 20 mM glutamine, 20  $\mu$ g/ml oligomycin and 1.2  $\mu$ g/ml antimycin A to prevent glutamate oxidation. Under these conditions glutamine and phosphate are at saturating concentrations [10]. For assays in intact cells, 0.2 ml of cell suspension, equivalent to  $40 \times 10^6$  cells, was used; after 6 min of incubation at 37°C the reaction was stopped by the addition of 0.5 ml of 0.4 N HCl. For assays in isolated mitochondria, about 50  $\mu$ g of total protein were used with 10 min of incubation. The KOH-neutralized mixture was centrifuged at  $16,000 \times g$  during 5 min. Finally, the glutamate formed was determined according to Lund [15]. To ensure PAG was indeed the enzyme assayed, the results of controls without phosphate were always subtracted [16]. Blanks with sample omitted were also run in parallel. Ehrlich ascites tumor cells and isolated mitochondria were counted with a Coulter Counter ZM (England). Protein concentrations were determined using a modified Lowry procedure [17].

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**Abbreviations:** GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAG, phosphate-activated glutaminase; SDS, sodium dodecyl sulphate.

### 2.3. RNA isolation and blot hybridization analysis

PAG mRNA levels were determined by Northern blot analyses. Total RNA from Ehrlich ascites tumor cells was isolated as described by Chomczynski and Sacchi [18]. The poly(A)<sup>+</sup> RNA fraction was enriched using the Fasttrack mRNA isolation kit from Invitrogen (San Diego, CA, USA). RNAs were fractionated on 1.5% agarose gels containing 0.8 M formaldehyde, transferred onto positively charged Nylon membranes (Boehringer, Mannheim, Germany), and then hybridized to the cDNA that corresponds to the coding sequence of rat kidney phosphate-activated glutaminase gene [19], kindly supplied by Prof. N.P. Curthoys (Colorado State University, USA). The cDNA was labelled using the Megaprime DNA labelling system with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham Ibérica, Spain). Hybridization was carried out at 42°C in 50% formamide, 5 × SSPE, 5 × Denhardt's and 20 µg/ml of denaturated salmon sperm DNA for 16–24 h. The membranes were successively washed for 20 min at 60°C with 2 ×, 0.5 ×, and 0.1 × SSC containing 0.1% SDS. Kodak X-OMAT AR films were exposed to the blots at -80°C with intensifying screens for 7–14 days. As an internal standard, the mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected, using a 1.3 kb GAPDH cDNA [20]. Autoradiograms were analyzed by transmittance densitometry, using a Hoefer GS 300 scanning densitometer (Hoefer Scientific, USA).

## 3. Results and discussion

Fig. 1 shows the capacity of intact Ehrlich ascites carcinoma cells for hydrolysing glutamine throughout tumor development. The results, expressed as either per mg of protein or per cell number, indicated that PAG is more active during the exponential phase of growth, with a subsequent decrease when the cells reach the stationary growth phase (Fig. 1 and Fig. 2A). It is noteworthy that even in tumor cells harvested in the stationary phase of growth, the glutamine-hydrolysing capacity is very high [21]. Increased specific activities of other glutamine-utilizing enzymes, particularly those involved in pyrimidine and purine biosynthesis, have also been described in experimental and human tumors [22]. Since in our experiments the glutamate formed in the absence of phosphate was always subtracted, the hydrolysis of glutamine in-

Table 1

Total protein content of intact cells and mitochondria during Ehrlich ascites carcinoma development

	Days after tumor transplantation				
	4	7	9	11	14
Cell	100	77.6 ± 1.3	73.0 ± 1.3	72.0 ± 5.2	82.2 ± 6.6
Mitochondria	100	76.0 ± 3.2	N.D.	36.0 ± 2.7	46.9 ± 1.3

Values at day 4 (152 ± 10 µg protein/10<sup>6</sup> cells and 2.1 ± 0.1 µg protein/10<sup>6</sup> mitochondria) are given as 100%. Values are means ± S.E.M. of three different determinations. Cells and mitochondria were counted and total protein determined as described in section 2. N.D., not determined.

deed reflects the phosphate-activated glutaminase reaction which is the first step in glutamine catabolism. Furthermore, the 'phosphate-independent' glutaminase activity was only appreciable in whole depleted cells, being negligible in isolated mitochondria (results not shown).

Phosphate-activated glutaminase has been described as a mitochondrial enzyme [4]; a similar profile to that showed for intact cells was obtained when PAG was assayed using isolated tumor mitochondria (Fig. 2B). Maximum activity was found in mitochondria isolated from cells in the exponential phase of growth and the activity decreased in mitochondria isolated from cells in the stationary phase of growth. The PAG activity was expressed as specific activity based on total protein content of mitochondria. In order to assess whether this observed decrease in PAG activity corresponds to a real change in the mitochondrial PAG enzyme, or whether it is the result of a protein change in the cell or in the mitochondria, total protein content was measured in both intact cells and mitochondria throughout tumor development. Table 1 shows that the total protein content decreased both in intact cells and isolated mitochon-

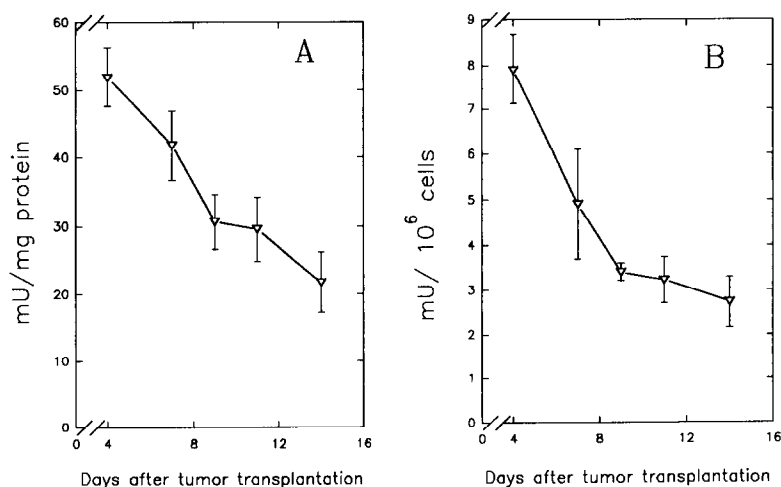


Fig. 1. Phosphate-activated glutaminase activities of intact Ehrlich ascites carcinoma cells during tumor development. Controls with phosphate omitted were always subtracted, as described in section 2. Values in mU (nmol/min) per mg of total protein (A) or per 10<sup>6</sup> cells (B) are the means of at least three different experiments ± S.E.M.

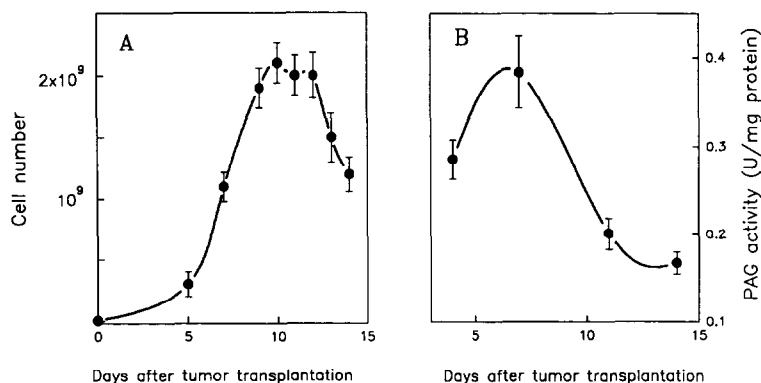


Fig. 2. (A) Growth curve for Ehrlich ascites carcinoma cells after the inoculation of  $5 \times 10^6$  cells (taken from [24]). (B) Phosphate-activated glutaminase activity in isolated mitochondria from Ehrlich cells at different days after tumor transplantation. Values are means of at least three different duplicate experiments  $\pm$  S.E.M.

dria from the 4th day after tumor transplantation. The results confirm that a real decrease in the PAG activity occurs when cell proliferation stops.

By only measuring activity, even in isolated mitochondria, it is not possible to establish a long-term regulation of PAG during tumor development unless a parallel increase in the respective mRNA levels is confirmed. Thus, the cell PAG mRNA levels at different days of tumor development should be analyzed. Autoradiograms of blot hybridization analyses of poly(A)<sup>+</sup> RNA were standardized with mRNA of glyceraldehyde-3-phosphate dehydrogenase, using a GAPDH cDNA rehybridized in the same filter (Fig. 3). A significant increase in PAG mRNA was obtained on the 7th day as compared with the mRNA of the 11th day. The profile of mRNA levels at different days throughout tumor development parallels the PAG activity; it seems that the expression of PAG is roughly correlated to the specific PAG activity. From its relative electrophoretic mobility, the size of the Ehrlich ascitic tumor glutaminase mRNA was estimated to be approximately 7.0 kb (Fig. 3). A very weak minor band corresponding to a mRNA of 3.4 kb was also detected. The relative sizes of tumor glutaminase mRNAs are in good agreement with those reported for rat kidney by Hwang et al. [23], who also observed two bands of 6.0 and 3.4 kb with the 6.0 kb band as the most prominent.

The presented results indicate that expression of phosphate-activated glutaminase, the first enzyme of the glutaminolytic process, is subject to long-term regulation during tumor development. In vivo, glutamine is supplied by the host tissues, namely liver, kidney and muscle [24]. Carrascosa et al. [21] reported that tumors elicit a specific response in the host nitrogen metabolism so that the whole organism is mobilized to increase circulating glutamine. This amino acid is avidly consumed by the tumor in such a way that, on the 7th day following tumor transplantation, glutamine was found neither in the ascitic liquid, the natural medium of Ehrlich carcinoma growth,

nor in the cells [25]. Thus, a positive gradient from host plasma to tumor cells is created. In this context, glutaminase could be considered the main pathway for nitrogen trapping by tumors. Although PAG activity is elevated in transformed cells, maximum expression occurs when the cells are actively dividing during the exponential phase of growth, coincident with the maximum nitrogen requirement.

Tumor cells apparently waste glucose and glutamine, since malignant tumors overcome their own needs. A continuous output of the end products, lactate, glutamate and ammonia, conform that tumor behaves as a powerful dissipative system. Crabtree and Newsholme [1,26] have theoretically justified the wasteful spreading of energy and nitrogen in very rapidly growing cells: a high rate of glutaminolysis is needed, not only for precursor provision per se, but for the sensitivity of the pathways involved in the biosynthetic processes; our re-

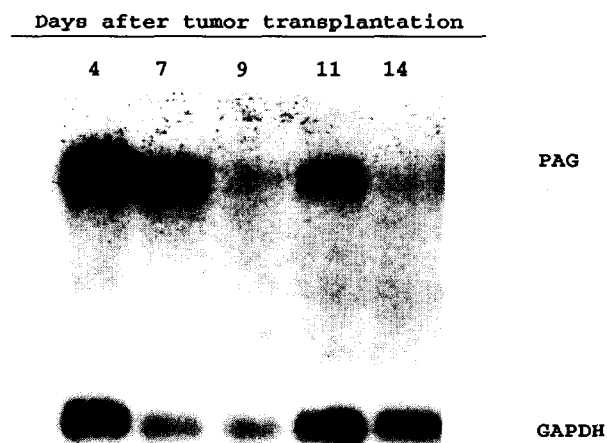


Fig. 3. Northern blot analysis of poly(A)<sup>+</sup> RNA from Ehrlich tumor cells growing 'in vivo' at different days after tumor inoculation. <sup>32</sup>P-labeled cDNA from rat kidney PAG was used as a probe. Experimental conditions are explained in section 2. The signal for control mRNA (GAPDH) is shown below the PAG autoradiogram, and it was obtained by rehybridization of the same filter.

sults seem to support this hypothesis. The experimental evidence here presented links the activity increase with a significant increase in the relative mRNA levels. The mechanisms responsible for the long-term regulation of tumor PAG will require further characterization; however, the results now reported will lead to a better understanding of the role of this key enzyme in neoplastic transformation.

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